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(54) Title: PROPAGATION AND INDUCIBLE DIFFERENTIATION OF HUMAN FETAL CENTRAL NERVOUS SYSTEM PROGENITOR CELLS

(57) Abstract

The present invention provides a process of preparing proliferating human fetal central nervous system progenitor or differentiated cells and cells prepared by that process. Proliferating progenitor cells are prepared by culturing human fetal, central nervous system tissue in a non-adherent culture container containing medium comprising serum and epidermal growth factor. A screening assay and *ex vivo* treatments using those cells are also provided.

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PROPAGATION AND INDUCIBLE DIFFERENTIATION OF HUMAN FETAL CENTRAL NERVOUS SYSTEM PROGENITOR CELLS

Description

Technical Field of the Invention

The present invention relates to processes and compositions for preparing human fetal central nervous system progenitor cells and differentiated neuronal and glial cells; as well as their use.

Background of the Invention

The mammalian central nervous system originates from cells of the embryonic neural tube. During development, these cells proliferate and differentiate into the diverse cell types of the mature central nervous system (CNS), including neurons and glia. Multipotential proliferating cells derived from fetal rat striatum, fetal rat cerebral cortex, post natal rat forebrain, chicken optic tectum and foetal and adult mouse striatum have been propagated *in vitro* and found capable of generating neurons and glia; long-term propagation of similar progenitor cells of human origin has not yet been described.

The regulation of proliferation and differentiation is not well understood. Certain factors influencing the fate of rodent CNS progenitor cells have recently been identified. Thus, epidermal growth factor (EGF) exerts a mitogenic action on low-density, dissociated cultures of adult and fetal mouse CNS tissue, yielding mixed cultures of neurons and astrocytes presumably arising from a population of multipotent progenitor cells (See PCT Publication No. WO93/01275). Neurotrophic effects of EGF, including the enhancement of survival and neurite outgrowth of postnatal rat striatal, cortical and cerebellar neurons in primary culture, have also been demonstrated. Insulin-like growth factors (IGF-I and IGF-II) have also been found to be growth promoting hormones. *In vitro* studies have demonstrated effects of IGF-I on neurons and glia, including stimulation of cell proliferation, DNA synthesis, oligodendrocyte development and myelination.

The present invention provides that epidermal growth factor, when added to a partially defined medium, allows the survival and proliferation of progenitor cells from human forebrain, including primordia of the cortex, striatum and basal forebrain. These progenitors can be maintained in a proliferating state for months. At any time, they can be induced to differentiate into neurochemically defined neuron and glia consistent with the cell types found in these forebrain areas in the mature state. Differentiated neurons derived from the progenitor cells display ligand-gated conductances characteristic of normal mammalian neurons.

Brief Summary of the Invention

In one aspect, the present invention provides a process of preparing a human fetal central nervous system progenitor cell comprising the steps of:

(a) culturing a population of cells from human fetal central nervous system tissue in a non-adherent culture dish containing proliferation culture medium that includes serum and an effective amount of epidermal growth factor; and

(b) maintaining the population of cells in the culture medium for a period of time sufficient for formation of a cellular mass that contains progenitor cells.

The central nervous system tissue can be from the spinal cord or the brain. Brain tissue can be from any specific region of the brain as exemplified by ventral forebrain tissue, cerebral cortex tissue or tissue from midbrain precursor.

In one embodiment, the effective amount of epidermal growth factor is from about 1 ng/ml to about 100 ng/ml of culture medium, more preferably from about 10 to about 50 ng/ml of culture medium and, even more preferably about 30 ng/ml of culture medium.

In a preferred embodiment, the proliferation culture medium further contains an effective amount of an insulin-like growth factor. The insulin-like growth factor is preferably insulin-like growth factor I and an effective amount of insulin-like growth factor I is from about 10 to about 100 ng/ml of culture medium and, more preferably about 40 ng/ml of culture medium. The proliferation culture medium can further contain transferrin, progesterone, putrescine, insulin and a selenite salt. An especially preferred proliferation medium is a 3:1 mixture of DMEM and F-12, supplemented in this manner.

The serum in the proliferation medium is preferably normal horse serum. Serum is preferably present in a concentration from about 1 volume percent to about 25 volumes percent, more preferably from about 2 to about 10 volumes percent and, even more preferably about 5 volumes percent.

In a preferred embodiment, the cell concentration is between about 10^2 and 10^4 cells per mm^2 of culture vessel surface and, more preferably about 2 x 10^3 cells per mm^2.

In another aspect, the present invention provides a human fetal central nervous system progenitor cell produced by a process of the present invention.

In another aspect, the present invention provides a process of preparing a differentiated, human central nervous system cell comprising the steps of:

(a) culturing a population of cells from human fetal central nervous system tissue in a non-adherent culture dish that contains a proliferation culture medium that comprises serum and an effective amount of epidermal growth factor;

(b) maintaining the population of cells in the culture medium for a period of time sufficient for formation of a cellular mass that contains progenitor cells; and

(c) inducing differentiation of the progenitor cells contained in the cellular mass.

In a preferred embodiment, inducing differentiation is accomplished by dissociating progenitor cells from the cellular mass, plating the dissociated cells onto adherent culture dishes and culturing the dissociated cells in a differentiation culture medium. In one embodiment, the differentiation medium contains serum such as fetal bovine serum and a minimal essential medium such as Dulbecco's MEM.

A differentiated cell produced by a process of the present invention can be a glial cell or a neuron including, but not limited to, a cholinergic neuron, a peptidergic neuron, a GABAergic neuron, a glutamatergic neuron or a catecholaminergic neuron.

Preferred embodiments for the process of producing a differentiated cell are the same as set forth above with regard to the process of preparing a progenitor cell.

The present invention also provides a differentiated human central nervous system cell produced by a process of this invention. That differentiated cell can be a glial cell or a neuron.

In yet another aspect, the present invention provides a screening assay for identifying substances that alter the function of a human central nervous system progenitor or differentiated cell, the process comprising the steps of:

(a) establishing a culture of proliferating human central nervous system progenitor cells or differentiated cells; and

(b) testing the ability of a substance to alter the function of those cells.

In a preferred embodiment, progenitor or differentiated cells are established in accordance with the processes set forth above. In one embodiment, testing is accomplished by exposing the cells to a substance suspected of altering function. Exposing is preferably accomplished by culturing the cells in a culture medium containing the substance suspected of altering function. In another embodiment, exposing is accomplished by transfecting the progenitor or differentiated cell with an expression vector that contains a polynucleotide that encodes the substance suspected of altering function, the expression vector driving expression of the substance in the cell.

In still yet another aspect, the present invention provides a composition for use in proliferating human fetal central nervous system progenitor cells comprising (a) a minimal essential medium; (b) serum: and (c) an effective amount of epidermal growth factor. In a preferred embodiment, the effective amount of epidermal growth factor is from about 1 ng/ml to about 100 ng/ml of medium, more preferably from about 10 to about 50 ng/ml of culture medium and, even more preferably about 30 ng/ml of culture medium.

A composition of the present invention can further comprise an effective amount of an insulin-like growth factor such as insulin-like growth factor I. An effective amount of insulin-like growth factor is preferably from about 10 to about 200 ng/ml of culture medium and, more preferably about 40 ng/ml of culture medium. The composition can further comprise transferrin, progesterone, putrescine, insulin and a selenite salt.

II. Human Fetal Central Nervous System Progenitor Cells

In one aspect, the present invention provides a process of preparing a human fetal central nervous system progenitor cell. That process comprises the steps of:

(a) culturing a population of cells from human fetal central nervous system tissue in a proliferation culture medium in a non-adherent culture dish containing serum and an effective amount of epidermal growth factor; and

(b) maintaining the population of cells in the culture medium for a period of time sufficient for formation of a cellular mass that contains progenitor cells.

As used herein, a central nervous system "progenitor" cell means an omnipotent cell that is capable of asymmetric cell division and produces daughter cells capable of differentiation into neurons or glial cells.

Cells used in a process of the present invention are obtained from a human fetus. Central nervous system tissue is typically obtained from the developing central nervous system of fetuses available from elective abortion. Central nervous system tissue is dissociated from the fetus using standard dissociation techniques well known in the art. By way of example, cells are physically dissociated from specific regions of the central nervous system by blunt dissection. Alternatively, cells are obtained by chemical means including treatment of tissue with enzymes such as trypsin, collagenase and the like.

As used herein, the phrase "central nervous system" means the brain and spinal cord or fetal tissue that gives rise to those tissues in the adult. Central nervous system cells, once dissociated from tissue, are typically purified using standard procedures well known in the art. Exemplary purification procedures are centrifugation of cells suspended in a physiological medium and passage of suspended cells through filters (e.g., pipettes) of various pore size. Preferred physiological media include isotonic solutions of NaCl,

balanced salt solutions and the like. A physiological medium is preferably calcium (Ca) and magnesium (Mg) free and can comprise enzymes such as trypsin and DNase to minimize clumping of cells. The existence of viable cells is confirmed by standard techniques such as ethidium bromide exclusion.

A population of cells for use in a process of the present invention can be obtained from any specific region of the human fetal central nervous system. Selection of a particular region will determine to a large extent the particular nature of differentiated cells obtained from formed progenitor cells. Exemplary specific regions are ventral forebrain, cerebral cortex, cerebellum, midbrain and brainstem.

Dissociated cells are suspended in a proliferation culture medium and plated onto a culture container having a non-adherent surface. As used herein, the term "non-adherent" or its grammatical equivalent indicates a surface coating to which cells will not adhere. Non-adherent culture containers are well known in the art and are commercially available.

A proliferation culture medium can be any minimal essential medium (MEM)known to support cell growth. Typically, a proliferation medium contains an isotonic level of balanced salts, nutrients such as amino acids, vitamins, and growth supporting factors such as transferrin, progesterone, putrescine, insulin and selenite. Exemplary proliferation media are Dulbecco's Minimal Essential Medium (DMEM) and Ham's F-12. In a preferred embodiment, a proliferation medium is a mixture of DMEM and F-12 in a 3:1 ratio. Media for use in a process of the present invention can be made or purchased from commercial sources.

A proliferation medium comprises an effective amount of epidermal growth factor (EGF). As used herein, "an effective amount" means that concentration of EGF that results in proliferation of progenitor cells. Means

for determining an effective amount are well known in the art. One skilled in the art can simply alter the concentration of EGF and determine the effects of such alteration on proliferation. An effective amount of EGF depends inter alia on the concentration of cells in the proliferation medium, the nutrient level of that medium and the absence or presence of other growth supporting factors in the medium. Where the concentration of cells is between about 10^2 and 10^4 cells per mm^2 and the medium is a 3:1 mixture of DMEM and F-12, an effective amount of EGF is preferably from about 1 ng/ml of medium to about 100 ng/ml of medium. More preferably, under those conditions, an effective amount of EGF is from about 10 ng/ml of medium to about 50 ng/ml of medium and, even more preferably from about 20 ng/ml of medium to about 40 ng/ml of medium.

The proliferation medium can further comprise an effective amount of an insulin-like growth factor (IGF). A preferred IGF is IGF-I. An effective amount of an IGF is determined in the same manner as set forth above with regard to EGF. Under the specific culture conditions set forth hereinafter in the examples, an effective amount of an IGF is from about 1 ng/ml of medium to about 100 μg/ml of medium. Preferably, the effective amount of an IGF is from about 10 ng/ml of medium to about 100 ng/ml of medium and, more preferably about 40 ng/ml of medium.

The concentration of cells in the proliferation medium (cell density) is selected to maximize proliferation. That concentration depends, as is well known in the art, on the nature of the proliferation medium (e.g., level of nutrients). Means for determining a cell concentration are well known in the art. In a preferred embodiment, where the population of cells is obtained from the ventral forebrain and the proliferation medium is a 3:1 mixture of DMEM and F-12, the concentration of cells is from about 10^2 to about 10^4 cells/mm^2. More preferably, cell concentration is from about 10^2 to about 5 x 10^3 cells/mm^2 and, more preferably about 2 x 10^3 cells/mm^2.

The proliferation medium used to prepare proliferating human fetal central nervous system cells contains normal serum. Normal serum can be derived from bovine, equine, chicken and the like. Normal horse serum is preferred. The concentration of serum can range from about 1 percent by volume to about 25 percent by volume. Preferably, the concentration of serum is from about 2 percent by volume to about 10 percent by volume and, more preferably about 5 percent by volume.

Cells are cultured in proliferation medium, under the conditions set forth above, for a period of time sufficient for formation of a cellular mass that contains progenitor cells capable of proliferation and differentiation. Culture conditions are those physiological conditions necessary for cell viability and include temperature, pH, osmolality and the like. Preferably, the temperature is from about 30°C to about 40°C, more preferably from about 35°C to about 39°C and, even more preferably about 37°C. The pH value is preferably from about a valueof about 6 to a value of about 8, more preferably from a value of about 6.5 to a value of about 7.5. and, even more preferably about 7.4. Osmolality is preferably from about 250 mosmols/liter to about 320 mosmols/liter, more preferably from about 270 mosmols/liter to about 310 mosmols/liter and, even more preferably from about 280 mosmols/liter to about 300 mosmols/liter. Cells in culture are fed as needed by replacing the proliferation medium with fresh medium containing the specified growth factors and serum. Typically, feeding occurs every 3-4 days.

Human fetal central nervous system cells, cultured as described above, form cellular masses that contain progenitor cells. Those cellular masses are typically spherical in nature. The progenitor cells in those masses can be dissociated and further propagated (e.g., subcultured) to form further masses of proliferating cells. Using a process of the present invention, human fetal progenitor cells can be propagated for extended periods of time (see the Examples hereinafter showing prpagation for at least four months in culture).

The propagation of progenitor cells from adult and fetal rodent central nervous system tissue has been reported (See, e.g., PCT Publication WO 93/01275). As reported hereinafter in the examples, human fetal central nervous system progenitor cells could not be propagated using the compositions and process reported in that publication.

The present invention also contemplates proliferating human fetal central nervous system progenitor cells prepared in accordance with a process of this invention. Those cells can be individual cells existing in a suspension culture or cells contained in cellular masses as set forth above. Thus, the present invention provides a culture of human fetal central nervous system progenitors cells capable of propagation in culture and differentiation.

A progenitor cell contemplated by the present invention can be derived from any specific region of human fetal central nervous system as set forth above.

III. Differentiated, Human Central Nervous System Cells

A human fetal central nervous system progenitor cell of the present invention can be induced to differentiate into neural and glial cells. Thus, the present invention contemplates a process of preparing such differentiated cells comprising inducing differentiation of progenitor cells of the present invention. A process of preparing a human central nervous system differentiated cell comprises the steps of:

(a) culturing a population of cells from human fetal central nervous system tissue in a non-adherent culture dish that contains proliferation culture medium that comprises serum and an effective amount of epidermal growth factor;

(b) maintaining the population of cells in the culture medium for a period of time sufficient for formation of a cellular mass that contains progenitor cells; and

(c) inducing differentiation of the progenitor cells contained in the cellular mass.

In a preferred embodiment, the proliferation medium further comprises IGF and, more preferably IGF-1. Preferred concentrations of IGF are the same as set forth above.

Differentiation can be induced using any means well known in the art. In a preferred embodiment, differentiation is induced by dissociating progenitor cells from the cellular mass, plating the dissociated cells onto an adherent culture dish and culturing the dissociated mass in a differentiation culture medium.

Progenitor cells are typically dissociated from the cellular mass using trituration with a pipette, usually after prior incubation with proteolytic enzymes. Culture containers having an adherent coating (a coating to which the cells adhere) are well known in the art. Exemplary adherent coatings include polyamines such as poly-L-lysine and poly-L-ornithine and extracellular matrix substances such as collagen, laminin and fibronectin. Culture containers having such coatings are commercially available.

The differentiation medium is any minimal essential medium that supports growth and differentiation. Exemplary and preferred such media are the same as set forth above. A preferred differentiation medium is DMEM. The differentiation medium can further comprise serum and, preferably fetal serum such as bovine fetal serum. The concentration of serum can range from about 1 percent by volume to about 25 percent by volume, preferably from about 5 percent by volume to about 15 percent by volume and, more preferably about 10 percent by volume. Growth factors may be present in the differentiation medium.

A human central nervous system differentiated cell prepared by a process of the present invention can be a neuron or a glial cell. More than one type of neuron can be prepared by a process of the present invention. The particular neuron type prepared depends inter alia upon the specific region of the central nervous system from which the progenitor cells are derived. Neuron types preparable by a process of the present invention include, but are not limited to, cholinergic neurons, peptidergic neurons, GABAergic neurons, glutamatergic neurons and catecholaminergic neurons.

The particular type of neuron prepared is determined using standard procedures well known in the art. Typically, differentiated cells are screened and identified using immunohistochemical techniques employing antibodies for known cell markers. The use of such immunohistochemical techniques is also used to identify differentiated glial cells. Antibodies against specific neural and glial cell markers are well known in the art. Exemplary cell markers are neuron-specific enolase (NSE), neurofilament (NF), glial fibrillary associated protein (GFAP), galactocerebroside (GalC), acetylcholine (ACh), dopamine (DA), epinephrine (E), norepinephrine (NE), histamine (H), serotonin or 5-hydroxytryptamine (5-HT), substance P (SP), human nerve growth factor receptor (NGF-R), choline acetyltransferase (ChAT), enkephalin (Enk), glutamic acid decarboxylase (GAD), tyrosine hydroxylase (TH), the neuronal/glial lineage marker, A2B5, dynorphin and aromatic amino acid decarboxylase (AADC). A detailed description of the use of immunochemical techniques to identify neuronal and glial cell markers is set forth hereinafter in the examples.

In one embodiment, the differentiation of human fetal central nervous system cells is regulated. Regulation is accomplished by adding defined growth factors and/or conditioned media to either or both of the proliferation and differentiation medium. Those growth factors can be used to direct differentiation toward specific cell types. Regulating the differentiation of specific cell types can also be accomplished as is well known in the art by

co-culturing with other cells types or transfecting cells with polynucleotides such that particular polypeptides (e.g., neural transmitters, enzymes) are expressed.

The present invention also contemplates differentiated human central nervous system cells as provided by a process of this invention. Those cells can exist as isolated and purified cells or as a culture of such cells. Differentiated neuronal and glial cells are contemplated by this invention.

IV. Screening Assays

Human central nervous system progenitor and differentiated cells of the present invention can be used in a variety of ways including screening assays for the identification of substances that affect the function of those cells and for ex vivo transplantation.

In yet another aspect, therefore, the present invention contemplates a process of screening substances for their ability to interact with a progenitor or differentiated human central nervous system cell of the present invention. Such a screening assay comprises the steps of providing a progenitor or differentiated cell of the present invention and testing the ability of selected substances to affect the function of that cell. In a preferred embodiment, providing a progenitor or differentiated cell is accomplished using a process set forth above.

Testing the ability of a selected substance to affect cell function is accomplished by exposing a culture of a progenitor or differentiated cells to a candidate substance suspected of affecting cell function, maintaining the cell under biological conditions and detecting the functional effects of that candidate substance.

Utilizing the methods and compositions of the present invention, screening assays are provided for the testing of candidate substances such as

inducers of differentiation, inhibitors of differentiation, drugs that are agonists or antagonists of cell receptors or membrane channels (e.g., Na channel, K channel, Ca channel, neurotransmitter receptors), toxins, drugs that affect membrane permeability to selective ions (e.g., Na, K, Cl, Ca), second messengers or gene expression and the like. A candidate substance is a substance which potentially can interact with or modulate, by binding or other intramolecular interaction, the metabolic or electrical properties of a progenitor or differentiated cell. Cells of the present invention are useful because of the difficulty in obtaining sufficient human central nervous system tissue for study.

With the availability of propagated progenitor and differentiated human cells, a screening assay of the present invention has several advantages over currently available assays. A major advantage is the availability of large amounts of uniform, nontransformed human central nervous systems cells. In addition, the investigator may now control the type of neuron or cell that is utilized in a screening assay. Specific neuronal cell types can be prepared expressed and their interaction with a substance can be identified. Other advantages include the availability of isolated neuron types previously difficult to obtain in tissue samples, and the obviation of finding a continuous supply of human material, particularly fetal material.

As is well known in the art, a screening assay provides a cell under conditions suitable for the testing of a candidate substance. These conditions include but are not limited to pH, temperature, tonicity, the presence of relevant co-factors and the like. The present invention provides culture conditions and compositions suitable for the prolonged culturing of both progenitor and differentiated human central nervous system cells.

In a typical screening assay for identifying candidate substances, cells are placed into a suitable culture medium (proliferation or differentiation). Candidate substances are added to the medium in convenient concentrations

and the interaction between the candidate substance and the cell is monitored. By comparing reactions which are carried out in the presence or absence of the candidate substance, one can then obtain information regarding the effect of candidate/receptor interaction on the cell's function.

Accordingly, it is proposed that this aspect of the present invention provides those of skill in the art with methodology that allows for the identification of candidate substances having the ability to modify the function of a human central nervous system progenitor or differentiated cell in one or more manners.

Where the candidate substance is a polypeptide, a progenitor or differentiated cell can be exposed to that substance by transfecting the cell with an expression vector that contains a polynucleotide that encodes that polypeptide. The expression vector drives expression of the polypeptide in the progenitor or differentiated cell. Means of transfecting central nervous system cells are well known in the art and include techniques such as calcium-phosphate- or DEAE-dextran-mediated transfection, protoplast fusion, electroporation, liposome mediated transfection, direct microinjection and adenovirus infection.

The most widely used method is transfection mediated by either calcium phosphate or DEAE-dextran. Although the mechanism remains obscure, it is believed that the transfected DNA enters the cytoplasm of the cell by endocytosis and is transported to the nucleus. Depending on the cell type, up to 90% of a population of cultured cells can be transfected at any one time. Because of its high efficiency, transfection mediated by calcium phosphate or DEAE-dextran is the method of choice for experiments that require transient expression of the foreign DNA in large numbers of cells. Calcium phosphate-mediated transfection is also used to establish cell lines that integrate copies of the foreign DNA, which are usually arranged in head-to-tail tandem arrays into the host cell genome.

In the protoplast fusion method, protoplasts derived from bacteria carrying high numbers of copies of a plasmid of interest are mixed directly with cultured mammalian cells. After fusion of the cell membranes (usually with polyethylene glycol), the contents of the bacteria are delivered into the cytoplasm of the mammalian cells and the plasmid DNA is transported to the nucleus. Protoplast fusion is not as efficient as transfection for many of the cell lines that are commonly used for transient expression assays, but it is useful for cell lines in which endocytosis of DNA occurs inefficiently. Protoplast fusion frequently yields multiple copies of the plasmid DNA tandemly integrated into the host chromosome.

The application of brief, high-voltage electric pulses to a variety of mammalian and plant cells leads to the formation of nanometer-sized pores in the plasma membrane. DNA is taken directly into the cell cytoplasm either through these pores or as a consequence of the redistribution of membrane components that accompanies closure of the pores. Electroporation can be extremely efficient and can be used both for transient expression of cloned genes and for establishment of cell lines that carry integrated copies of the gene of interest. Electroporation, in contrast to calcium phosphate-mediated transfection and protoplast fusion, frequently gives rise to cell lines that carry one, or at most a few, integrated copies of the foreign DNA.

Liposome transfection involves encapsulation of DNA and RNA within liposomes, followed by fusion of the liposomes with the cell membrane. The mechanism of how DNA is delivered into the cell is unclear but transfection efficiencies can be as high as 90%.

Direct microinjection of a DNA molecule into nuclei has the advantage of not exposing DNA to cellular components such as low-pH endosomes. Microinjection is therefore used primarily as a method to establish lines of cells that carry integrated copies of the DNA of interest.

The use of adenovirus as a vector for cell transfection is well known in the art. Adenovirus vector-mediated cell transfection has been reported for various cells including cells of the central nervous system.

Expression vectors that drive expression of a polypeptide typically include (if necessary) an origin of replication, a promoter, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences. Promoters specific to the cell being studied are preferred. Thus, where a neuron is transfected, a preferred promoter is a neurofilament or neuron-specific enolase promoter. Other promoters that have particular utility in neural cells are promoters associated with the production of neurotransmitters such as ACh, E, NE, 5-HT and dopamine.

For use in mammalian cells, the control functions on the expression vectors are often derived from viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, Cytomegalovirus and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication.

An origin of replication can be provided with by construction of the vector to include an exogenous origin, such as can be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV, CMV) source, or can be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

A transfected progenitor or differentiated human central nervous system cell can also be used in a screening assay to test for candidate substances that affect cell function. In addition, such a cell can be used for propagating human neurotropic viruses or other fastidious pathogens, for research, development of vaccines, or other purposes.

V. Ex Vivo Treatment

Human progenitor and differentiated cells of the central nervous system of the present invention provide a source of human cells for use in ex vivo transplantation into the human brain. Ex vivo transplantation is becoming a treatment of choice for certain pathological disorders of the central nervous system.

Diseases such as Alzheimer's Disease, Huntington's Chorea and Parkinson's Disease have been linked to the degeneration of neurons in specific locations in the brain and the inability of the brain region to synthesize and release neurotransmitters and other substances vital to neuron function. As the population at risk for such diseases increases there is an increasing need for new and improved ex vivo treatment regimens.

The use of drugs and other pharmacological agents has limited utility for treatment of central nervous system pathologies. Drugs administered via typical parenteral routes of administration have difficulty in reaching target tissues in the brain because of transport problems across the blood-brain barrier. Administration directly into the brain or spinal cord gives rise to potential infection in those tissues. Further, tolerance to drugs is often associated with long-term use of those drugs. By way of example, despite partial restoration of dopaminergic activity seen in Parkinson's patients treated with L-dopa, those patients often become tolerant to the effects of L-dopa, and thus require increasing dosages to maintain the beneficial effects of that drug.

Neurological tissue grafting (ex vivo therapy) of tissue into the central nervous system has been used to overcome some of the problems associated with drug therapy. Typically, ex vivo treatment is used to replace the source of a particular substance where the intrinsic source of that substance has been destroyed as a result of dysfunction. By way of example, Parkinson's Disease is associated with a loss of dopamine producing cells in

the brain. The transplantation of cells that produce dopamine can be used to partially restore brain function.

Because of a shortage of human brain tissue for transplantation, ex vivo treatment often involves the use of cells and tissue from other sources. Exemplary such tissues and cells are adrenal medulla (adrenal chromaffin cells), neurons from the adult peripheral nervous system (PNS), chromaffin cells and fetal ventral mesencephalon from non-human species and immortalized cell lines derived either by transformation of normal cells or by culturing cells with altered growth characteristics.

There are problems associated with the use of such cells for transplantation into human CNS. For example, first, non-human cells give rise to an immune response. Second, the use of non-neural cells compromises the ability of the grafted cells to functionally integrate with the CNS. Third, transformed cells may form tumors in the patient's brain.

The use of human cells can obviate problems of immune rejection. Human brain tissue from juveniles or adults, however, have limited survival potential following transplantation. For this reason, human fetal CNS tissue is preferred for ex vivo therapy. Although the successful use of such fetal tissue has been reported (Perlow, *et al.*, *Science* 204: 643-647, 1979; Lindvall, *et al.*, *Science* 247: 574-577, 1990; Freed, *et al.*, *Arch. Neurol.* 47: 505-512, 1990), the use of human fetal tissue is severely limited because of the available supply of such tissue and the ethical problems associated with obtaining human fetal tissue.

There is a need, therefore, for a reliable source of unlimited numbers of human fetal CNS cells for neurotransplantation, which cells are capable of differentiating into neurons. The present invention provides such a reliable source of human fetal central nervous system tissue or cells.

The present invention therefore provides a process of ex vivo treatment of human central nervous system disorders comprising administering to patients in need of such treatment, a human fetal central nervous system progenitor or differentiated cell of the present invention. Human fetal progenitor cells or differentiated cells of the present invention can also, in combination with routine immunosuppressive medication, be administered to any animal with abnormal neurological or neurodegenerative symptoms obtained in any manner, including those obtained as a result of chemical or electrolytic lesions, as a result of experimental aspiration of neural areas, or as a result of aging processes.

Cells are delivered throughout any affected neural area. Cells are administered to a particular region using any method which maintains the integrity of surrounding areas of the brain, preferably by injection cannula.

The selection of which cells to use in transplantation depends upon the desired purpose of the treatment. Where, for example, it is desirable to restore levels of a particular substance (e.g., a neurotransmitter such as dopamine) to a region of the brain, cells that produce that substance are selected. Means for identifying particular cell types are set forth hereinbefore. In a preferred embodiment, cells administered to the particular region of the brain form neuronal or synaptic connections with neighboring neurons, and maintain contact with glial cells which may form myelin sheaths around the neuron's axon.

Survival of the transplanted tissue is determined using standard non-invasive procedures well known in the art. Such procedures include computerized axial tomography (CAT or CT scan), nuclear magnetic resonance or magnetic resonance imaging (NMR or MRI) and positron emission tomography (PET) scans. Functional integration of the cells into the host's neural network is assessed by examining the effectiveness of grafts on restoring various functions.

In addition to restoring lost function (e.g., neurotransmitter production), cells of the present invention can be used to deliver drugs or other substances to specific regions of the central nervous system. In accordance with this embodiment, a progenitor or differentiated cell is transfected with an expression vector containing a polynucleotide that encodes that substance, the expression vector driving expression of the substance in the transfected cell. Means for transfecting cells are set forth hereinbefore. Cells expressing the desired substance are transplanted to a desired location in the brain.

The following examples are illustrative of particular embodiments of the present invention and are not limiting of the specification and claims in any way.

EXAMPLES

EXAMPLE 1: Isolation of Human Fetal CNS Cells

Human fetal brain tissue was obtained from routine therapeutic abortions performed at the Victoria General Hospital in Halifax, Nova Scotia, with informed consent and approval of appropriate institutional ethical review boards. Evacuated tissue fragments from 6-8 week post-conception fetuses, as staged according to the developmental atlas of England (1983), were collected directly into sterile ice-cold isotonic saline with heparin (10μg/ml). Ventral forebrain was dissected under stereomicroscopic observation in a laminar flow containment hood, and transferred to a solution of 0.05% (w/v) trypsin (Type XIII, Sigma) in calcium- and magnesium-free Hank's Balanced Salt Solution (CMF-HBSS) for 20 minutes at 37°C.

This incubation was followed by 4 washes with 0.01% deoxyribonuclease (DNase I, sigma) in complete HBSS. The tissue was then dissociated to a suspension of single cells in 0.01% DNase I in CMF-HBSS by repeated gentle passage through fire-polished Pasteur pipettes of decreasing bore size. Viability of the dissociated cells was assessed by exclusion of ethidium bromide, after incubation in a solution of acridine

orange and ethidium bromide (1μg/ml each in HBSS). Cells were counted in a hemocytometer using standard fluorescein and rhodamine fluorescence filters to detect acridine orange- and ethidium bromide-containing cells, respectively.

EXAMPLE:2 Preparation of Progenitor Cells

Cells were plated at 2.0 x 10^3 viable cells per mm^2 in 24-well uncoated Nunc tissue culture dishes. Progenitor cell culture medium (PCM) was composed of a 3:1 mixture of Dulbecco's Minimum Essential Medium (DMEM:Gibco) and Ham's F-12 (Gibco), with 5% (v/v) horse serum (Gibco), insulin (Sigma, 10 μg/ml), transferrin (Sigma, 200 μg/ml, progesterone (Sigma, 40 mM), putrescine (Sigma, 200 μM) and sodium selenite (Sigma, 60 nM). This PCM was further supplemented with either EGF (Upstate Biochemicals, 20ng/ml) (PCM-E) or EGF plus IGF-I (Upstate Biochemicals, 100ng/ml) (PCM-EI). Cells were fed at 3-4 day intervals throughout the experiment with PCM, PCM-E or PCM-EI. At intervals of 30-60 days *in vitro* (DIV), resulting spherical cell masses were dissociated as above, and cells replated at a density of 2 x 10^3 viable cells per mm^2.

Human fetal ventral forebrain tissue plated in PCM-E at a density of 2 x 10^3 cells per mm^2 on uncoated tissue culture plastic consistently formed spherical cellular masses 0.2-1.5 mm in diameter after 30-60 DIV. This mass formation was not seen with cells plated at higher density (>5 x 10^3 cells per mm^2) on uncoated tissue culture plastic, nor with cells plated on poly-L-lysine-coated dishes at densities of ≤ 2 x 10^2 per mm^2. Cells grown in PCM without additional additives did not produce growth of these masses, while the formation of these masses was greatly enhanced in PCM-EI.

Immunohistochemical staining revealed nestin-immunoreactive cells within the cellular masses when these masses were plated, undissociated, directly onto poly-L-lysine-coated plates for 48 hours. The masses attached loosely to the plate during this time period via small numbers of processes.

After dissociating and replating the cells of these masses, 'secondary' cell masses formed; growth of secondary cells masses was less rapid than that of primary cell masses. After a further 30-60 DIV, secondary masses were similarly dissociated to produce 'tertiary' masses of proliferating cells. Such passage could be repeated at least 2 times.

EXAMPLE 3: Differentiation of Human CNS Cells

To induce differentiation, spherical proliferating cell masses were dissociated as above, and the resulting cell suspension plated onto poly-L-lysine (Sigma)-coated dishes in DMEM with 10% fetal bovine serum (FBS). For cell cycle analysis, spherical masses of proliferating cells were, while still in PCM, exposed to 1μg/ml bromodeoxyuridine (BrdU; Sigma), which is incorporated into the nuclear DNA of proliferating cells, for 6, 12, 18, 24, 36, 48, 60 or 72 hours. Cell masses were then dissociated, and the resulting cell suspension plated onto poly-L-lysine-coated plates in DMEM with 10% FBS. After 12 hours, these cells were fixed with 4% paraformaldyhyde in 0.1M phosphate buffer, pH 7.4, for 20 minutes at room temperature. For phenotypic analysis of progenitor cell progeny, some cells exposed to BrdU for 48 hours were grown on coated plates in DMEM with 10% FBS for up to ten days before fixation and staining.

Immunocytochemical staining was carried out as previously described, using well-characterized antibodies specific for BrdU (Amersham), neurofilament 200 (NF; Sigma), glial fibrillary associated protein (GFAP; Sigma and DAKO), choline acetyltransferase (ChAT; Chemcion), glutamic acid decarboxylase (GAD; Calbiochem), tyrosine hydroxylase (TH; Eugene Tech), substance P (Sub-P, Eugene Tech), the neuronal/glial lineage marker A2B5 (Boehringer Mannheim), (Raff et al., 1983) and nestin-129 (Tohyama et al., 1992; kindly supplied by R. McKay, NIH). Also, cells were incubated with FITC labelled tetanus toxin (FITC-TT; List Biologicals Laboratories, Inc.).

Briefly, cells were fixed for 20 minutes at room temperature by replacing culture medium with 4% paraformaldyhyde in 0.1 M phosphate buffer, pH 7.4. Cells to be stained for BrdU were incubated with 0.1% (w/v) DNase in 0.01 M phosphate buffer prior to primary antibody treatment, to increase antibody access to incorporate BrdU. Dilutions of primary antibodies or TT in 0.1 M phosphate buffer containing 1% normal serum were as follows: rabbit anti-GFAP (1:100), rabbit anti-NF (1:200), mouse anti-BrdU (neat), rabbit anti-ChAT (1:1000), rabbit anti-GAD (1:200), rabbit anti-TH (1:1000), rabbit anti-Sub-P (1:1000), mouse anti-A2B5 (1:100), rabbit anti-nestin-129 (1:2000), and FITC-TT (200 μg/ml).

Bound antibodies were localized by immunofluorescence, using Texas red-conjugated sheep anti-mouse (Amersham) and fluorescein-conjugated donkey anti-rabbit (Amersham) immunoglobulins diluted 1:30. To determine the proportion of cells incorporating BrdU, all BrdU-labelled and unlabelled cells were counted in 10 randomly-selected fields of view in each well using a 40x objective (n=6) with epifluorescence and differential interference-contrast optics. BrdU-labelled cells were expressed as percent of total cells.

Tertiary cell masses, collected at least 75 days after initial primary plating, were redissociated and plated as single cells onto poly-L-lysine coated tissue culture dishes. After 5 DIV, these cells were fixed and stained immunohistochemically to evaluate their cytochemical phenotype; cells immunoreactive for NF, ChAT, GFAP, Sub-P, TH, A2B5, GAD and TT were evident. When these cells were derived from cellular masses incubated with BrdU for 48 hours immediately prior to dissociation, cells of each cytochemically-identified group were found with BrdU-labelled nuclei.

To establish the fraction of cells actively replicating, tertiary cell masses were incubated with BrdU for various periods immediately prior to dissociation and replating; plated cells were fixed and stained immunocytochemically to detect BrdU-labelled cells. The percentage of cells

incorporating BrdU into their nucleus increased with increasing incubation times from 12 to 36 hours; with longer incubations, the proportion of labelled nuclei remained unchanged at approximately 80% of the total.

Cell cultures used for electrophysiological experiments were grown on poly-L-lysine coated glass coverslips for 22 to 70 DIV. At the time of the experiment, a single coverslip was transferred to a glass-bottomed Perspex recording chamber mounted on the stage of a Nikon Diaphot inverted microscope. Cultures were observed using phase-contrast optics, and were continuously perfused with a salt solution of the following composition (in mM): NaCl, 149; KCl, 3.25; $CaCl_2$, 2; $MgCl_2$, 2; D-glucose, 11; tetrodotoxin, 0.0003; Hepes buffer, pH 10. The pH of the perfusate was adjusted to 7.25 using NaOH, and osmolarity was adjusted to 350 mOsm using sucrose. Drugs were diluted from concentrated stock solution into the same sl at solution which, in the case of experiments involving N-methyl-D-aspartate (NMDA), was supplemented with glycine (10 μM) but lacked $MgCl_2$. Conventional patch pipettes were formed from borosilicate glass using a Mechanex BBCH puller; they were not additionally fire polished or treated to reduce capacitance.

The patch pipettes were filled with a solution containing (in mM): CsF, 120; CsCl, 10; Hepes,10; EGTA, 10; $CaCl_2$, 0.5. The pH was adjusted to 7.25 with CsOH and osmolarity to 330 mOsm with sucrose. Whole-cell currents were recorded from cultured neurons using a List EPC-7 patch clamp amplifier. Pipette seal resistances were not routinely measured, but were generally in excess of 100 GΩ. Pipette capacitance transients were cancelled prior to rupturing the membrane but no additional capacitance neutralization or series resistance compensation was applied. Drugs were applied to localized regions of the culture by fast perfusion form a double-barrelled pipette assembly. The internal diameter of each of the perfusion tubes was approximately 375 μm and they were positioned close to the bottom of the dish and approximately 300 μm from the cell.

Differentiated neurons derived from tertiary spheres responded electrophysiologically to the excitatory amino acid analogs NMDA, α-amino-3-hydroxy-5-methyl-isoxazolepropionate (AMPA) and kainate in a manner qualitatively similar to that of rat cortical neurons. Thus, NMDA (30 μM) responses required the presence of glycine, were associated with a substantial increase in membrane noise and decayed during exposure to the agonist (mean peak amplitude: 1.1 ± 0.5 nA, n=6). AMPA responses consisted of an initial rapidly desensitizing component which decayed to a steady-state current; as noted previously for rat cortical neurons in culture, the steady state current was maximal (315 ± 90 pA, n=13) at 100 μM whereas the amplitude of the peak desensitizing component continued to increase up to concentrations of 1 mM. Kainate responses were typically non-desensitizing and appeared to be maximal (1.46 ± 0.5 nA, n=6) at 1 mM. Responses to combined applications of NMDA (30 μM) and glycine (10 μM) were completely blocked by the competitive NMDA antagonist, CGS195755 (30 μM, n=4). Likewise, combined applications of NMDA (30μM, n=4). Interestingly, preliminary recordings from less mature human neuronal cultures (8-14 DIV) revealed significantly smaller responses to the same excitatory amino acid agonists (50 pA for 100 μM NMDA or AMPA, 300 pA for 1 mM kainate) either in comparison to rat cortical neurons of comparable maturity or compared to the more mature human cultures of 22-70 DIV reported above.

These results demonstrate that EGF and IGF-I, when added to a partially defined medium, permit the survival and continued proliferation *in vitro* of progenitor cells from the fetal human forebrain for at least 4 months. Such cultured progenitor cells remain in an undifferentiated state, expressing nestin, until they are stimulated to differentiate.

Here, differentiation was induced by changing culture conditions to FBS-supplemented medium in culture dishes coated to promote attachment. A wide range of differentiated cells can in this way be produced, including

neurons (as indicated by expression of neurofilament) of several neurochemical classes (catecholaminergic as indicated by TH, peptidergic as indicated by Sub-P, cholinergic as indicated by ChAT and inhibitory GABAergic as indicated by GAD expression) and astrocytes (as indicated by GFAP expression). These differentiated neurons can be maintained *in vitro* for at least 15 weeks when cultured on glial feeder layers, and display normal and medically-significant receptors by electrical recording criteria. All these differentiated cell types derived from progenitor cells of forebrain origin are also present in the normal adult forebrain. EGF and IGF-I also permitted the formation of proliferating cell masses from human fetal ventral mesencephalic cells.

It should be noted that the EGF-supplemented defined culture medium used by Reynolds and Weiss, 1992 did not induce the formation of progenitor cells from either the forebrain or the ventral mesencephalon of human fetal origin.

To rule out the possibility that the histochemically identified cells were primary cells that had persisted in culture, rather than differentiated offspring of progenitor cells, progenitor cells were incubated with BrdU at a concentration that would be incorporated into the DNA but not affect proliferation. The cells were then induced to differentiate, and stained for both BrdU and particular neurochemical markers. Double-labelled cells, immunoreactive for BrdU and for ChAT, substance P, TH or GAD were observed, establishing that all of these cells could be produced by progenitor cells replicating in culture prior to terminal differentiation.

Double-labelled astrocytes (immunoreactive both for BrdU and for GFAP) were also routinely observed.

Cell cycle analysis indicates that the progenitor cells proliferate with an approximate 36 hour cycle, and that approximately 70% of the clustered

cells are mitotically active. Mitotic division of true stem cells would yield one differentiated cell as well as another stem cell; alternatively, division of uncommitted progenitor cells could yield two other progenitor cells. The latter would yield a large proportion of proliferating cells while the former only a small fraction of the total. The data suggest that the cells described here are progenitors rather than stem cells, since progenitor cells can be shown to exhibit differentiation potential as well as a capacity of asymmetric cell division. Cells unlabelled with BrdU during the 48 hour incubation period have presumably ceased proliferation, for unknown reasons that may include nutritive constraints, spontaneous differentiation, or effects of the BrdU itself.

Trophic factor support for the proliferation and differentiation of neuron cells has been demonstrated by others. The ability of EGF and IGF-I to support proliferation of these undifferentiated progenitor cells supports the possibility that these mitogenic growth factors play a role in normal development of the fetal human forebrain. IGF-I mRNA has been shown to be present in the human fetal CNS and similarly EGF receptors have been localized throughout the adult human brain.

Examples have been included to illustrate preferred modes of the invention. Certain aspects of the examples are described in terms of techniques and procedures found or contemplated by the present inventors to work well in the practice of the invention. These examples are exemplified through the use of standard laboratory practices of the inventor. In light of the present disclosure and the general level of skill in the art, those of skill will appreciate that the examples are intended to be exemplary only and that numerous changes, modifications and alterations can be employed without departing from the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A process of preparing a human fetal central nervous system progenitor cell comprising the steps of:
   (a) culturing a population of cells from human fetal central nervous system tissue in a non-adherent culture dish containing proliferation culture medium that comprises serum and an effective amount of epidermal growth factor; and
   (b) maintaining the population of cells in the culture medium for a period of time sufficient for formation of a cellular mass that contains progenitor cells.

2. The process of claim 1 wherein the central nervous system tissue is brain.

3. The process of claim 1 wherein the proliferation culture medium further contains an effective amount of an insulin-like growth factor.

4. The process of claim 1 wherein the serum is normal horse serum.

5. A culture of proliferating human fetal central nervous system progenitor cells.

6. A process of preparing a differentiated human central nervous system cell comprising the steps of:
   (a) culturing a population of cells from human fetal central nervous system tissue in a non-adherent culture dish that contains proliferation culture medium that comprises serum and an effective amount of epidermal growth factor;
   (b) maintaining the population of cells in the culture medium for a period of time sufficient for formation of a cellular mass that contains progenitor cells; and

(c) inducing differentiation of the progenitor cells contained in the cellular mass.

7. The process of claim 6 wherein inducing differentiation is accomplished by dissociating cells in the cellular mass, plating the cells onto adherent culture dishes and culturing the dissociated mass in a differentiation culture medium.

8. The process of claim 6 wherein the differentiated cell is a neuron.

9. The process of claim 6 wherein the differentiated cell is a glial cell.

10. A culture of differentiated human central nervous system cells.

11. The culture of claim 10 wherein the differentiated cells are neurons.

12. The culture of claim 10 wherein the differentiated cells are glial cells.

13. A screening assay for identifying substances that alter the function of a human fetal central nervous system progenitor cell, the process comprising the steps of:

(a) establishing a culture of proliferating human fetal central nervous system progenitor cells; and

(b) testing the ability of a substance to alter the function of the progenitor cells.

14. The assay of claim 13 wherein establishing the culture is accomplished by:

(a) culturing a population of cells from human fetal central nervous system tissue in a non-adherent culture dish containing proliferation culture medium that comprises serum and an effective amount of epidermal growth factor; and

(b) maintaining the population of cells in the proliferation culture medium for a period of time sufficient for formation of a cellular mass that contains progenitor cells.

15. The assay of claim 13 wherein testing is accomplished by exposing the progenitor cells to a substance suspected of altering function.

16. The assay of claim 15 wherein exposing is accomplished by culturing the progenitor cells in a culture medium containing the substance suspected of altering function.

17. The assay of claim 15 wherein exposing is transfecting the progenitor cell with an expression vector that contains a polynucleotide that encodes the substance suspected of altering function, the expression vector driving expression of the substance in the progenitor cell.

18. A screening assay for identifying substances that alter the function of a human central nervous system differentiated cell, the process comprising the steps of:

(a) establishing a culture of differentiated human central nervous system differentiated cells; and

(b) testing the ability of a substance to alter the function of the differentiated cells.

19. The assay of claim 18 wherein testing is accomplished by exposing the differentiated cells to a substance suspected of altering function.

20. The assay of claim 18 wherein exposing is accomplished by culturing the differentiated cells in a culture medium containing the substance suspected of altering function.

21. The assay of claim 18 wherein exposing is transfecting the differentiated cell with an expression vector that contains a polynucleotide that encodes the substance suspected of altering function, the expression vector driving expression of the substance in the differentiated cell.

22. A composition for use in proliferating human fetal central nervous system progenitor cells comprising:
    (a) a minimal essential medium;
    (b) serum: and
    (c) an effective amount of epidermal growth factor.

23. The composition of claim 22 further comprising an effective amount of an insulin-like growth factor.

INTERNATIONAL SEARCH REPORT

Inter[redacted] Application No
PCT/CA 95/00445

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 01275 (WEISS S. ET AL) 21 January 1993 cited in the application see page 40 - page 44	1-12
A	WO,A,94 10292 (NEUROSPHERES) 11 May 1994 see the whole document	1-23
A	WO,A,94 09119 (NEUROSPHERES) 28 April 1994 see the whole document	1-23
A	EXPERIMENTAL NEUROLOGY 128 (1). 1994. 34-40. , VON VISGER J R ET AL 'Differentiation and maturation of astrocytes derived from neuroepithelial progenitor cells in culture.'	1-23

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCIENCE, vol. 255, 27 March 1992 LANCASTER, PA US, pages 1707-1710, REYNOLDS A. ET AL. 'Generation of Astrocytes from Isolated Cells of the Adult Mammalian Central Nervous System' see the whole document -----	1-23

INTERNATIONAL SEARCH REPORT

Inter[redacted] Application No
PCT/CA 95/00445

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9301275	21-01-93	AU-A-	2242592	11-02-93
		CA-A-	2113118	21-01-93
		EP-A-	0594669	04-05-94
		FI-A-	935929	02-02-94
		JP-T-	6509225	20-10-94
		NO-A-	940056	03-03-94
		WO-A-	9416718	04-08-94
WO-A-9410292	11-05-94	AU-B-	5367694	24-05-94
		EP-A-	0669973	06-09-95
		FI-A-	952022	27-04-95
		NO-A-	951617	27-04-95
		WO-A-	9416718	04-08-94
WO-A-9409119	28-04-94	AU-B-	5147493	09-05-94
		CA-A-	2147162	28-04-94
		EP-A-	0664832	02-08-95
		FI-A-	951677	07-04-95
		NO-A-	951378	07-04-95
		WO-A-	9416718	04-08-94